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Conversion of Cyclodextrin Glycosyltransferase into a Starch Hydrolase by Directed Evolution: The Role of Alanine 230 in Acceptor Subsite +1^{†,‡}

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ABSTRACT: Cyclodextrin glycosyltransferase (CGTase) preferably catalyzes transglycosylation reactions, whereas many other α -amylase family enzymes are hydrolases. Despite the availability of three-dimensional structures of several transglycosylases and hydrolases of this family, the factors that determine the hydrolysis and transglycosylation specificity are far from understood. To identify the amino acid residues that are critical for the transglycosylation reaction specificity, we carried out error-prone PCR mutagenesis and screened for *Bacillus circulans* strain 251 CGTase mutants with increased hydrolytic activity. After three rounds of mutagenesis the hydrolytic activity had increased 90-fold, reaching the highest hydrolytic activity ever reported for a CGTase. The single mutation with the largest effect (A230V) occurred in a residue not studied before. The structure of this A230V mutant suggests that the larger valine side chain hinders substrate binding at acceptor subsite +1, although not to the extent that catalysis is impossible. The much higher hydrolytic than transglycosylation activity of this mutant indicates that the use of sugar acceptors is hindered especially. This observation is in favor of a proposed induced-fit mechanism, in which sugar acceptor binding at acceptor subsite +1 activates the enzyme in transglycosylation [Uitdehaag et al. (2000) *Biochemistry* 39, 7772–7780]. As the A230V mutation introduces steric hindrance at subsite +1, this mutation is expected to negatively affect the use of sugar acceptors. Thus, the characteristics of mutant A230V strongly support the existence of the proposed induced-fit mechanism in which sugar acceptor binding activates CGTase in a transglycosylation reaction.

The α -amylase family, or glycoside hydrolase family 13 (1), is a large family of starch processing enzymes (2, 3). The $(\beta/\alpha)_8$ -barrel fold of the catalytic domain, the catalytic site residues, and the α -retaining bond cleavage mechanism are conserved in this family (4, 5), but the product and reaction specificity vary widely (6).

Cyclodextrin glycosyltransferase (CGTase)¹ is a member of this family that forms circular α -(1,4)-linked oligosaccharides composed of six, seven, or eight glucose residues (α -, β -, and γ -cyclodextrin, respectively). It consists of five domains (A–E); domains A and B constitute the catalytic domains and domain E is involved in raw starch binding (7, 8), whereas the functions of domains C and D are not known. After binding of the substrate across several sugar binding

subsites (labeled –7 to +2; Figure 1), the α -(1,4)-glycosidic bond between subsites –1 and +1 is cleaved to yield a covalent glycosyl-enzyme intermediate (5) that is bound at the donor subsites (–1, –2, –3, etc.) (Figure 2) (5). In the next step of the reaction an acceptor molecule binds at acceptor subsite +1 and cleaves the glycosyl-enzyme bond. In the cyclization reaction the nonreducing end of the covalently bound sugar is used as the acceptor to yield a cyclodextrin (Figure 2). CGTase may also use water or a second sugar molecule as acceptor, which results in a hydrolysis or a disproportionation reaction, respectively (Figure 2).

The relative efficiencies of the hydrolysis and transglycosylation reactions are determined by the nature of the acceptor used in the second half of the reaction and thus by the properties of the acceptor subsites. CGTase has a clear preference for glucosyl acceptors, as its transglycosylation activities are much higher than the hydrolysis activity (9). Previously, we have shown that mutations at acceptor subsite +2 can change CGTase into a starch hydrolase (10–12), whereas mutations at other subsites have much smaller effects on the hydrolysis/transglycosylation ratio (13–15). To explain the transglycosylation reaction specificity, it has been suggested that binding of a sugar, but not water, in acceptor subsite +1, activates CGTase for the second half of the reaction (16).

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[‡] Coordinates for the A230V mutant CGTase have been deposited with the Brookhaven Protein Data Bank (ID code 1PEZ).

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¹ Abbreviations: CGTase, cyclodextrin glycosyltransferase; BC251, *Bacillus circulans* strain 251; *Tabium*, *Thermoanaerobacterium thermosulfurigenes* strain EM1; EPS, 4-nitrophenyl- α -D-maltoheptaoside-4–6-*O*-ethylidene.

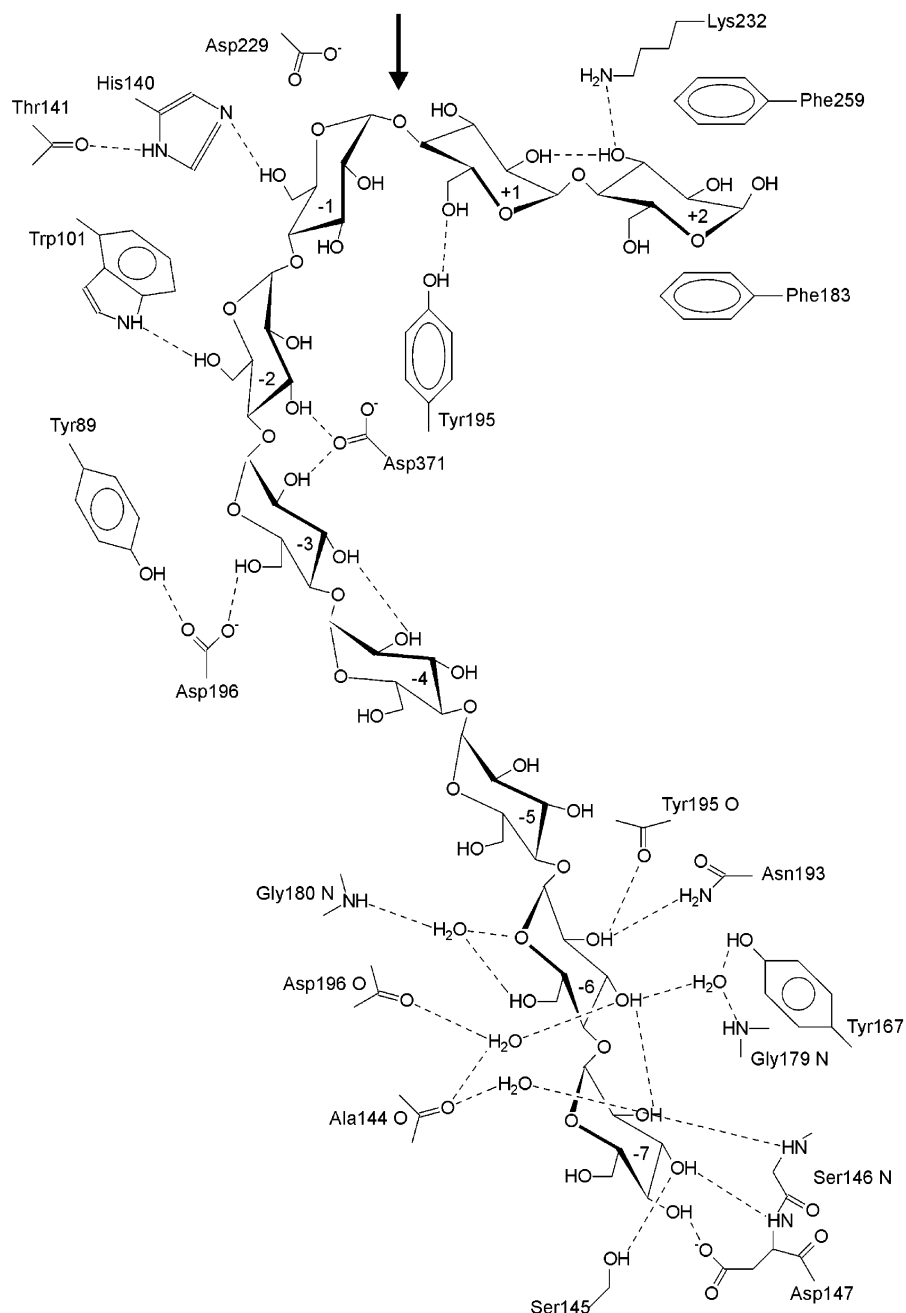


FIGURE 1: Schematic overview of the interactions between a maltononaose substrate and the substrate binding cleft of *B. circulans* CGTase. The arrow indicates the scissile bond between subsites -1 and $+1$; hydrogen bonds are shown as dashed lines. Arg47 and Asn94 do not interact with uncleaved substrate, but they do interact with the reaction intermediate and the product γ -cyclodextrin, respectively (16, 41). Phe183 and Phe259 have hydrophobic stacking interactions with the sugar rings. For clarity not all interactions at the -2 , -1 , and $+1$ subsites are shown. The figure has been adapted from ref 5.

To identify the amino acid residues that restrict the hydrolytic activity of CGTase and that are thus important for the transglycosylation reaction specificity of the enzyme, we randomly introduced mutations in *Bacillus circulans* strain 251 (BC251) CGTase and selected the variants displaying increased hydrolytic activity. Several mutated residues were identified, but the major finding was that the A230V mutation strongly enhanced the hydrolytic activity of CGTase and converted the enzyme into a starch hydrolase.

EXPERIMENTAL PROCEDURES

Structure Determination. Crystals of BC251 CGTase mutant A230V were grown from 60% v/v 2-methyl-2,4-

pentanediol, 100 mM HEPES buffer, pH 7.5, and 5% w/v maltose (17). Data were collected at 100 K on an in-house MARCCD system (MarUSA Inc., Evanston, IL) with a diameter of 165 mm and using Cu K α radiation from a BrukerNonius FR591 rotating-anode generator equipped with Osmic mirrors. Processing was done with DENZO and SCALEPACK (18). The structure of CGTase liganded with maltotetraose (PDB code 1CXF) with all waters and sugars removed was used as the starting model. Refinement was done with CNS (19). Ligands were placed in sigmaA-weighted $2F_o - F_c$ and $F_o - F_c$ electron density maps using the program O (20). Data and refinement statistics are given in Table 1. The atomic coordinates and the structure factors

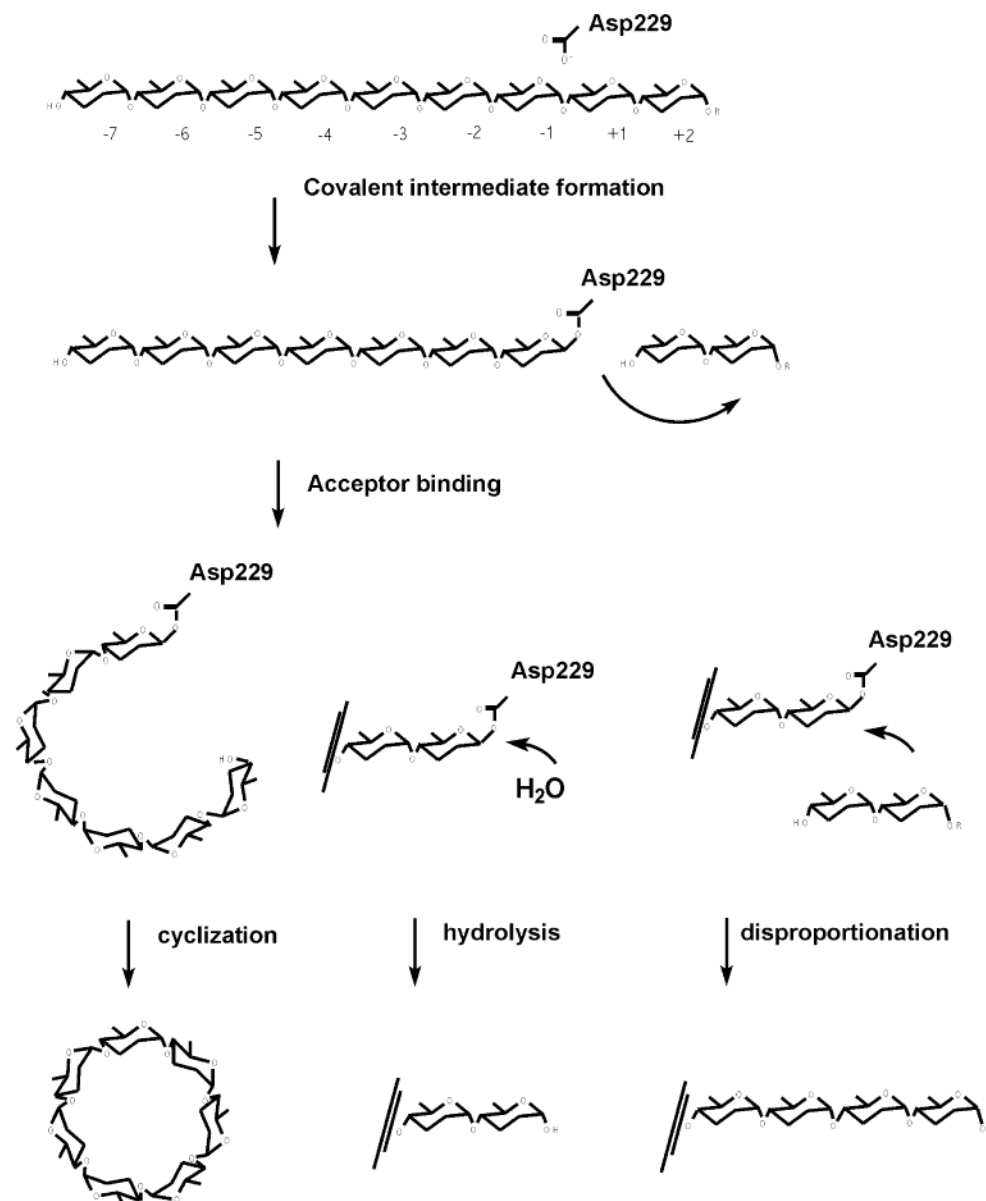


FIGURE 2: Schematic representation of the reactions catalyzed by CGTase. After bond cleavage a covalently bound reaction enzyme-glucosyl intermediate is formed. In the second step of the reaction the reaction intermediate is transferred to an acceptor molecule. In the cyclization reaction the terminal OH-4 group of the covalently linked oligosaccharide is used as acceptor, whereas water or a second sugar is used as acceptors in the hydrolysis and disproportionation reactions, respectively. This figure has been adapted from ref 23.

of mutant A230V have been deposited with the Protein Data Bank (code 1PEZ; www.rcsb.org).

Bacterial Strains and Plasmids. *Escherichia coli* MC1061 (21) was used for DNA manipulations, and *Bacillus subtilis* DB104A (22) was used for protein production. The plasmids pDP66k- (7) and pCScgt-tt (23), with the *cgt* genes of BC251 and *Thermoanaerobacterium thermosulfurigenes* strain EM1 (Tabium), respectively, were used for mutagenesis and protein production. Plasmid-carrying strains were grown on LB medium (24) at 37 °C in the presence of kanamycin, 50 or 6 µg/mL for *E. coli* or *B. subtilis*, respectively. When appropriate, potato starch (1.5% w/v) was added to LB agar plates to identify colonies expressing a starch degrading CGTase. Transformation of *B. subtilis* was done according to Bron (25).

Production and Purification of CGTase Proteins. CGTase proteins were produced using 2 L shake flasks with 0.5 L of LB medium supplemented with 10 g/L casamino acids. After

24 h of growth the culture was centrifuged (4 °C, 30 min, 10 000g). The CGTase proteins were purified from the culture supernatants by affinity chromatography using a 10 mL α-cyclodextrin–Sephacrose 6FF column (Pharmacia, Sweden). After being washed with 10 mM sodium acetate buffer (pH 5.5), bound CGTase was eluted with the same buffer containing 10 mg/mL α-cyclodextrin to obtain 1–5 mg of protein with a purity of about 95%. This purity was high enough to obtain crystals for three-dimensional structure determination. Protein concentrations were determined using the Bradford reagent from Bio-Rad (München, Germany) and a bovine serum albumin as standard.

Site-Directed Mutagenesis. Mutations and restriction sites were introduced into pDP66k- and pCScgt-tt as described (7, 11) and verified by DNA sequencing. The *Xho*I and *Kpn*I restriction sites introduced into pDP66k- resulted in V6S and A678G mutations (near the N- and C-termini, respectively), which had no measurable effect on the

Table 1: Data Collection Statistics and Quality of the *B. circulans* CGTase Mutant A230V

A230V	
data collection	
space group	P2 ₁ 2 ₁ 2 ₁
cell axes <i>a</i> , <i>b</i> , <i>c</i> (Å)	117.6, 109.7, 65.7
resolution range (Å)	25.0–2.32
total no. of observations	356544
no. of unique reflections	36647
completeness (%) ^a	97.8 (95.2)
$\langle I/\sigma(I) \rangle^a$	21.8 (4.6)
<i>R</i> _{merge} (%) ^a	6.0 (27.1)
refinement statistics	
no. of amino acids	686 (all)
no. of Ca ²⁺ ions	3
active site ligand	maltose
average <i>B</i> -factor	23.7
final <i>R</i> -factor (%) ^a	15.5 (19.2)
final free <i>R</i> -factor (%) ^a	19.9 (22.8)
rmsd ^b from ideal geometry	
bond lengths (Å)	0.005
angles (deg)	1.3
dihedrals (deg)	24.4
improper dihedrals (deg)	0.83

^a Highest resolution shell in parentheses. ^b rmsd = root mean square deviation.

cyclization, disproportionation, and hydrolysis activities of BC251 CGTase (data not shown). The primers used were as follows: F1 (*Xho*I), 5'-GCGCCGGATACCTCGAGTTC-CAACAAGCAAATTTTC-3', and R1 (*Kpn*I), 5'-CCAATTCACGTTAATGGTACCGGTGCCGCTGGACGG-3'; F21L, 5'-ATCTATCAAATTTTGACCGACAGGTTT-3'; R47W, 5'-ACGAACCTCTGGCTGTATTGC-3'; N94S, 5'-TCCGCGGTGAACAGCACGGCCTAT-3'; A245T, 5'-TTTATGGCTACCGTCAACAAC-3'; Q320L, 5'-GTGGATGACCTGGTGACGTTTC-3'; A357T, 5'-GGCGTCCCCACCATTTATTAC-3'; V660A, 5'-GGATCCACCGCCACGTGGGAA-3'; A231V, 5'-ATACGTCTAGATGTTGTAAAACATATG-3'. Mutated nucleotides and restriction sites are underlined.

Saturation Mutagenesis. Ala230 of BC251 CGTase was replaced by all 19 other amino acid residues. The mutations were introduced using the site-directed mutagenesis procedure, as described above, using the primer 5'-ATCCGCATGGATNNSGTGAAGCATATG-3' (A230X). N is A + G + C + T, S is G + C, and X is any amino acid residue.

Error-Prone PCR Mutagenesis. The BC251 *cgt* gene was amplified from pDP66k- with the primers F1 and R1. PCR mixtures (50 µL) contained 1× Taq DNA polymerase buffer, 1 mM MgSO₄, 0–1 mM MnCl₂, 0.6 mM each of dNTP, 0.07 µM each of primer, 20 ng of template, and 2.5 units of Taq DNA polymerase (Roche). PCR reactions were performed for 25 cycles: 30 s at 94 °C, 40 s at 54 °C, and 2 min at 72 °C. The PCR products were restricted with *Xho*I and *Kpn*I, and the resulting fragment (2016 bp) was extracted from agarose gel (QIAquick Gel Extraction Kit; Qiagen) and cloned in pDP66k-, replacing the wild-type *cgt* gene. The *Xho*I site is 12 nucleotides beyond the sequence coding for the export signal of CGTase (CGTase is an extracellular enzyme), and the *Kpn*I site is 30 nucleotides before the stop codon of the gene.

DNA Sequencing. Cycle sequencing (26) was performed on double-stranded DNA using the Thermo sequence fluorescent primer cycle sequence kit (Amersham Pharmacia Biotech AB), and the reactions were run on the Pharmacia

ALF-Express sequencing machine at BMTC (Groningen, The Netherlands). Error-prone PCR mutants were double-strand sequenced (six sequence reactions per mutant).

Selection of CGTase Variants with Increased Hydrolytic Activity. Ligation mixtures of the error-prone PCR products and the plasmid pDP66k- were transformed to *E. coli* MC1061 and plated on LB agar plates, and the resulting colonies were transferred to 200 µL of LB medium in 96-well microtiter plates using the Q-pix (Genetix, New Milton Hampshire, U.K.). After overnight incubation (750 rpm), 25 µL of each culture was transferred to a second microtiter plate containing 25 µL of bacterial protein extraction reagent (Pierce, Rockford, IL) per well to lyse the cells. Subsequently, 200 µL of 1% (w/v) soluble starch (Lamers and Pleuger, Wijnegem, Belgium) in 10 mM sodium citrate buffer (pH 6.0) was added, and the microtiter plates were incubated at 50 °C for 2 h in an oven. The amount of reducing sugars formed was measured using an adapted version of the Nelson–Somogyi assay (27, 28). Forty microliters of the reaction was added to 40 µL of solution D in polypropylene microtiter plates. After the microtiter plates were sealed with a polypropylene lid, they were incubated in an oven at 100 °C for 30 min. After being cooled to room temperature, 160 µL of solution E was added, allowing color development within a few minutes. Microtiter plates were screened visually, or the absorbance at 525 nm was measured. Solution D consisted of 25 mL of solution A (25 g of Na₂CO₃, 25 g of NaK tartrate, and 200 g of Na₂SO₄ in 1 L of demi-water) and 1 mL of solution B (30 g of CuSO₄·5H₂O and 0.2 mL of sulfuric acid in 200 mL of demi-water). Solution E consisted of 15.6 g of (NH₄)₆Mo₇O₂₄·4H₂O and 13.1 mL of sulfuric acid in 950 mL of demi-water plus 1.9 g of Na₂HAsO₄·7H₂O dissolved in 50 mL of demi-water.

Enzyme Assays. CGTase proteins were produced and purified as described (29). All enzyme assays were done in 10 mM sodium citrate buffer (pH 6.0) at 50 or 60 °C for BC251 and *Tabium* CGTase, respectively. Cyclization activities were determined by incubating 0.1–0.5 µg of enzyme/mL with 2.5% (w/v) partially hydrolyzed potato starch (Paselli SA2; Avebe, Foxhol, The Netherlands). The amount of β-cyclodextrin formed was measured with phenolphthalein (30). The disproportionation activity was determined as described (13, 31) using 0.1 µg of enzyme/mL, 1 mM 4-nitrophenyl-α-D-maltoheptaoside-4-6-*O*-ethylidene (EPS; Megazyme, County Wicklow, Ireland), and 10 mM maltose as donor and acceptor substrates, respectively. The wild-type CGTase has *K_M* values for the donor (EPS) and acceptor (maltose) substrates of 0.22 and 0.83 mM, respectively (9). The hydrolysis activity was determined by measuring the increase in reducing power upon incubation of 0.5–5 µg of enzyme/mL with 1% (w/v) soluble starch (Lamers & Pleuger, Wijnegem, Belgium) (29). Substrate affinity values are not reported for the hydrolysis and cyclization reactions since, at the low starch substrate concentrations needed, the amount of reducing power or cyclodextrin formed is too low for reliable activity measurements. Low starch concentrations are needed because BC251 CGTase has a high affinity for starch (<0.5 mg/mL) (9).

Structure Comparison. Three-dimensional structures were displayed and compared using the Swiss-PdbViewer, version 3.7 (b2) (32), and the program O (20). Figures were made using the Swiss-PdbViewer and Pov-Ray for Windows,

Table 2: CGTase Mutants with Increased Hydrolytic Activity Selected after Random Mutagenesis and Their Cyclization and Hydrolysis Activities^a

enzyme	additional mutations	cyclization [$\mu\text{mol}/(\text{min}\cdot\text{mg})$]	hydrolysis [$\mu\text{mol}/(\text{min}\cdot\text{mg})$]	disproportionation [$\mu\text{mol}/(\text{min}\cdot\text{mg})$]	overall activity [$\mu\text{mol}/(\text{min}\cdot\text{mg})$]
wild type ^b	V6S/A678G	266	3	970	1239 (100) ^d
first round					
1	F259S	52	40	468	1068 (86)
2	A230V	13	72	<10	95 (8)
3	ND ^c	26	19	11	56 (5)
4	ND	45	14	38	97 (8)
5	ND	30	26	26	82 (7)
6	A230V/V660A	14	70	<10	94 (8)
7	ND	113	12	1005	1130 (91)
8	F259S, N299D	47	45	524	616 (50)
9	ND	58	12	282	352 (28)
10	ND	29	36	49	114 (9)
11	A230V/A672G	14	70	<10	94 (8)
12	A230V/I215V	14	66	<10	90 (7)
13	ND	44	25	117	186 (15)
14	A230V	15	68	<10	93 (8)
15	F259S	48	43	475	566 (46)
16	A230V	14	71	<10	95 (8)
17	ND	50	32	424	506 (41)
18	ND	26	33	27	86 (7)
19	A230V/T514A	9	57	<10	76 (6)
20	F259S	49	42	483	574 (46)
21	A230V/K655E	11	72	<10	93 (8)
22	ND	39	15	339	393 (32)
second round					
6-1	F21L	29	174	<10	213 (17)
6-2	F21L	28	175	<10	213 (17)
6-3	A245T/A357T	30	163	<10	203 (16)
6-4	F21L	28	172	<10	210 (17)
third round					
6-2-1	N94S/N8S	28	255	<10	293 (23)
6-2-2	Q320L	32	246	<10	288 (23)
6-2-3	N94S/N8S	30	260	<10	300 (24)
6-2-4	N94S	31	267	<10	308 (25)
6-2-5	N94S	32	272	<10	314 (25)
6-2-6	R47W	13	250	<10	273 (22)
6-2-7	ND	19	186	<10	215 (17)
6-2-8	ND	18	222	<10	250 (20)

^a The activities were measured using purified proteins. ^b Contains V6S/A678G mutations but has wild-type activities. ^c ND, not determined.^d Numbers in parentheses indicate the relative overall activity compared to the wild-type enzyme.

version 3.1g. The BC251 CGTase structures used were entries 1CDG (17) and 1CXK (5) from the Protein Data Bank (33).

RESULTS

Error-Prone PCR Conditions. Optimal error-prone PCR mutagenesis conditions were determined by amplifying the BC251 *cgt* gene at 10 different MnCl_2 concentrations. The PCR products were cloned in plasmid pDP66k-, transformed into *E. coli*, and plated on LB plates complemented with starch. Only colonies expressing a starch-degrading CGTase form a halo around the colony. The percentage of halo-forming colonies decreased with increasing MnCl_2 concentrations (Figure 3), indicating that the number of inactivating mutations increased with higher MnCl_2 concentrations. A MnCl_2 concentration of 0.2–0.3 mM was chosen as the optimal error-prone PCR condition, with about 90% of the (mutant) CGTase clones obtained at this MnCl_2 concentration retaining starch-degrading activity (Figure 3).

Selection of CGTase Variants with Increased Hydrolytic Activity. About 12000 clones were assayed for hydrolytic activity in the first round of mutagenesis and selection. The MnCl_2 concentrations used in the PCRs were 0.2 mM (6000

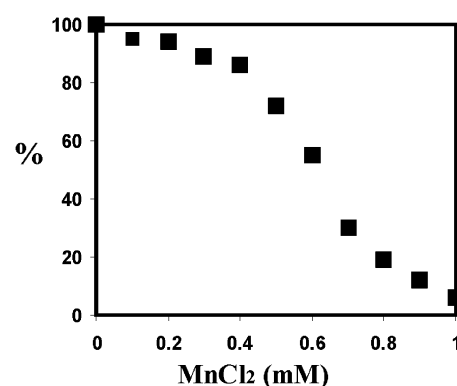


FIGURE 3: Percentage of clones that expressed an active starch-degrading CGTase (on LB starch plates) as a function of the MnCl_2 concentration used during PCR amplification of the BC251 *cgt* gene.

clones) and 0.3 mM (6000 clones). Twenty-two clones had a significantly increased hydrolytic activity. Plasmid DNA was isolated from each positive clone and subsequently used to produce and purify the encoded mutant proteins. All 22 mutant CGTases had higher hydrolytic and lower cyclization activities than wild-type CGTase (Table 2). Some mutants had even a higher hydrolytic than cyclization activity.

The hydrolytic activity of CGTase was further enhanced in a second and third round of mutagenesis using 0.25 mM MnCl₂ during PCR amplification. Mutants 6 and 6-2 were used as PCR templates in the second and third round of mutagenesis, respectively; these variants had the highest hydrolytic activity after their rounds of mutagenesis (Table 2). About 10000 clones each were assayed for hydrolytic activity in the second and third rounds of mutagenesis, resulting in four and eight clones with increased hydrolytic activity, respectively. Plasmid DNA of the positive clones was isolated, and the encoded proteins were produced and purified. The mutant CGTases identified in the second round had much higher hydrolytic activities than the parent mutant 6 (Table 2). Surprisingly, the cyclization activities had also increased (Table 2), although we selected for increased hydrolytic activity. The hydrolytic activity had further increased after the third round of mutagenesis, whereas the cyclization activity was unaffected or lowered compared to the parent mutant 6-2 (Table 2). Thus, a mutant CGTase with an almost 10-fold reduced cyclization activity and a 90-fold increased hydrolytic activity was obtained in only three rounds of mutagenesis and selection.

DNA Sequencing. Of the 34 selected CGTase variants with enhanced hydrolytic activity, 22 were subjected to nucleotide sequencing (2016 bp). This revealed 41 nucleotide substitutions and 31 amino acid mutations, of which 15 amino acid mutations were different (Table 2). Additional stop codons were not found, which was in agreement with the wild-type size of all selected mutant proteins on SDS-PAGE (data not shown). The mutants had one to three nucleotide mutations (1.9 on average) per round of mutagenesis, which resulted in one or two amino acid mutations (1.4 on average). The variations in mutation frequency were small (<10% at the DNA and the amino acid level) for the individual rounds of mutagenesis and the different MnCl₂ concentrations used (0.2, 0.25, and 0.3 mM). The substitutions A → G, T → C, C → T, and G → A were observed most frequently, whereas A → C, T → A/G, C → G, and G → T mutations were not found. The frequent observation of T → C and C → T mutations is due to the selection applied, as these changes were required to obtain the amino acid mutations causing the increased hydrolytic activity.

Mutations Enhancing Hydrolytic Activity. The 12 mutants displaying the highest hydrolytic activity after the first round of mutagenesis had either a F259S (four times) or an A230V (eight times) mutation, with or without a second mutation (Table 2). The second round of mutagenesis yielded a F21L mutation (three times) and once the combination A245T/A357T (Table 2), in addition to the A230V/V660A mutations. After the third round of mutagenesis the mutations N8S, R47W, N94S, and Q320L were identified, although N8S was identified in combination with N94S only (Table 2). Of the mutations with clearly enhanced hydrolytic activity, three mutations were in residues located in the substrate binding cleft at subsite -3 (Arg47), subsite +1 (Ala230), and subsite +2 (Phe259) (Figure 1), and one mutation occurred in a residue that has no direct interactions with substrates (Phe21).

Site-Directed Mutants. Of the 15 different amino acid mutations identified, seven mutations (N8S, I215V, N299D, T514A, K655E, V660A, and A672G) were only found in combination with a second mutation (A230V, F259S, or

Table 3: Cyclization, Disproportionation, and Hydrolysis Activities of (Mutant) BC251 and *Tabium* CGTases^a

CGTase	cyclization [μmol/ (min·mg)]	disproportionation [μmol/ (min·mg)]	hydrolysis [μmol/ (min·mg)]	overall activity [μmol/ (min·mg)]
BC251				
wild type ^b	266	970	3	1239 (100) ^f
F21L	196	952	10	1158 (93)
R47W	182	1305	15	1502 (121)
N94S	243	796	2	1041 (84)
A230V ^c	13	<10 ^e	72	95 (8)
A245T	260	934	3	1197 (97)
F259S ^c	52	468	40	1068 (86)
Q320L	251	976	5	1232 (99)
A357T	230	701	3	934 (75)
V660A	265	954	3	1222 (99)
<i>Tabium</i>				
wild type	240	510	54	804 (100)
A231V	10	<10 ^e	138	158 (20)
F260E ^d	19	21	177	217 (27)
A231V/F260E	8	59	21	88 (11)

^a The activities were measured using purified proteins. ^b Contains V6S/A678G mutations but has wild-type activities. ^c F259S and A230V are mutants 1 and 2, respectively, from Table 2. ^d Mutant F260E has been described in ref 10. ^e A230V and A231V, however, efficiently hydrolyzed the substrate of this reaction. ^f Numbers in parentheses indicate the relative overall activity compared to the wild-type enzyme.

N94S). Comparison of the single A230V, F259S, and N94S mutants with the double mutants showed that these seven mutations made no additional contribution to hydrolysis activities (Table 2). For mutant V660A this was confirmed; it had activities similar to those of wild-type CGTase (Table 3).

To analyze the individual effects of the other eight mutations (F21L, R47W, N94S, A230V, A245T, F259S, Q320L, and A357T), they were introduced as single mutation in wild-type CGTase. It appeared that N94S, A245T, Q320L, and A357T hardly altered the hydrolytic activity, whereas F21L, R47W, A230V, and F259S clearly caused an increased hydrolytic activity compared with wild-type CGTase (Table 3). In addition, these eight mutations lowered the cyclization and disproportionation activities, except mutant R47W, which had a higher disproportionation activity (Table 3). In particular, mutant A230V drastically lowered the cyclization and disproportionation activity. Thus, several residues (Phe21, Arg47, Ala230, and Phe259) important for the low hydrolytic activity of CGTase were identified.

Saturation Mutagenesis. Since error-prone PCR mutagenesis cannot introduce all possible mutations at a single position (34), the position of Ala230 was also investigated with saturation mutagenesis. Five hundred clones were assayed for hydrolytic and cyclization activity. Most clones (70%) had at most 5% of wild-type cyclization activity, whereas none of them had an increased cyclization activity, showing the importance of this residue for the cyclization reaction. Forty clones (8%) had a strongly increased hydrolytic activity. Eight of them were randomly selected for sequencing; all of them contained the A230V mutation. Thus, the mere change of an alanine to a valine side chain at position 230 is sufficient to strongly increase the hydrolytic activity and decrease the cyclization activity of CGTase.

***Tabium* CGTase.** Mutation A230V was also introduced into *Tabium* CGTase (A231V, *Tabium* CGTase numbering),

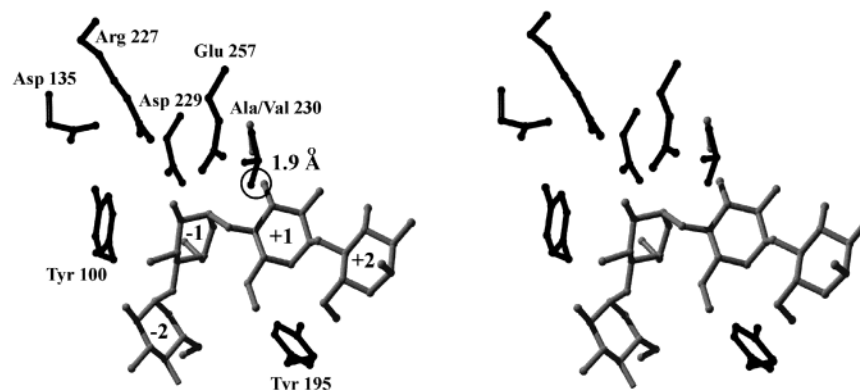


FIGURE 4: Stereo picture of a close-up view of the A230V mutant structure (black) superimposed on the structure of *B. circulans* D229N/E257Q CGTase with bound maltonaose (gray) (5). For clarity only the substrate binding subsites -2 to $+2$ are shown. The valine side chain of mutant A230V would form a close contact (1.9 Å) with the O3 atom of the glucose in acceptor subsite $+1$ of the maltonaose structure (the alanine side chain is at 3.2 Å).

as this CGTase has a relatively high hydrolytic activity compared to most other CGTases (35). Similar to the effects in BC251 CGTase, the A231V mutation strongly reduced the cyclization and increased the hydrolytic activity of *Tabium* CGTase (Table 3). In an attempt to further enhance the hydrolytic activity of this CGTase, the double mutant A231V/F260E was constructed, since until now *Tabium* mutant F260E had the highest hydrolytic activity ever described for a CGTase (11). However, the double mutant had an even lower hydrolytic activity than wild-type *Tabium* CGTase (Table 3), indicating that the two single mutations are mutually exclusive and not additive.

Structure. The three-dimensional structure of A230V CGTase (mutant 6) has a maltose ligand bound in the donor subsites -2 and -1 . In addition, a maltotetraose sugar is bound at the maltose-binding site 1 (17), located on the E-domain of the enzyme. The tetrasaccharide could be either a degradation product of the α -cyclodextrin used for the purification of the enzyme, or it could be the product of transglycosylation reactions on the maltose used for the crystallization. The three additional mutations present in mutant 6 (V660A, V6S, and A678G; the latter two were a result of the cloning procedure; see Experimental Procedures) did not affect the protein backbone conformation within the error limits.

Superimposing the A230V structure on CGTase with a bound maltonaose molecule (occupying subsites -7 to $+2$) (5) shows that the Val230 side chain would form a close contact (1.9 Å) with the O3 atom of the glucose residue in acceptor subsite $+1$ (Figure 4). Rotating the Val230 side chain away from the carbohydrate would result in an energetically unfavorable side chain conformation. Nevertheless, the interference with the substrate appears not severe enough to completely abolish carbohydrate binding at the $+1$ acceptor subsite, since this mutant is still able to process glycosidic bonds (Table 3).

DISCUSSION

The aim of this study was to identify residues that determine the transglycosylation reaction specificity of CGTase. Previously, we already identified by site-directed mutagenesis two phenylalanine residues at acceptor subsite $+2$ (Figure 1) as being very important for the hydrolysis/transglycosylation specificity of CGTase (10, 11). To determine whether there are more residues essential for the

transglycosylation reaction specificity of CGTase, we applied random mutagenesis and screened for clones with enhanced hydrolytic activity.

Phe259 Is Important for CGTase Reaction Specificity. Using error-prone PCR mutagenesis, we found that the F259S mutant has a strongly increased hydrolytic activity. This mutation was already known to strongly increase the hydrolysis activity of BC251 CGTase (10). The identification of the mutation F259S in our random mutagenesis and selection approach thus was an excellent control of the adequacy of the method. This result once again emphasized the importance of Phe259 for the transglycosylation reaction specificity of CGTase, as shown before (11, 12, 36).

Phe21 Limits the Hydrolytic Activity of CGTase. Phe21 is not a conserved CGTase residue and has no direct interactions with substrates. Nevertheless, mutation F21L strongly enhanced the hydrolytic activities of mutant 6 (A230V/V660A) and, to a lesser extent, of wild-type CGTase. Phe21 is surrounded by several conserved CGTase residues, including Tyr100 and His327, that interact with substrates in subsite -1 (10). Mutation studies have demonstrated the importance of Tyr100 and His327 for catalytic activity (37–39). Since the leucine side chain is somewhat smaller, mutation F21L may alter the conformation of Tyr100 and His327, which may have an effect at subsite -1 and at the conformation of the reaction intermediate. Thus, Phe21 was identified as a residue important for the reaction specificity of CGTase.

Mutations of Gln320 and Ala245/Ala357 Increase the Hydrolytic Activity of the A230V Mutants. The hydrolytic activities of mutants 6 (A230V/V660A) and 24 (F21L/A230V/V660A) are enhanced by the A245T/A357T and Q320L mutations, respectively. Although mutant Q320L displays some increased hydrolytic activity on its own, the single site mutations A245T and A357T have no effect on hydrolysis (Table 3). Thus, these mutations have only a significant effect in combination with other mutations. How they precisely affect the reaction specificity is not understood, as they are more than 10 Å away from Ala230 and Phe21. Moreover, they are also more than 10 Å away from the substrate binding cleft.

Subsite -3 Is Important for CGTase Reaction Specificity. BC251 CGTase structures have shown that oligosaccharides in subsite -3 are bound by Arg47, Tyr89, Asn94, Asp196,

and Asp371 (Figure 1) (16, 40, 41). The importance of these five residues for the cyclization reaction/product specificity of CGTases has been demonstrated by mutation studies (14, 38, 42). The identification of R47W and N94S mutations in mutant CGTases with enhanced hydrolytic activity in our random mutagenesis study shows again the importance of subsite -3 for the activities of CGTase, in agreement with previous studies.

Ala230 Is Essential for the Transferase Specificity of CGTase. In BC251 CGTase the Ala230 side chain is located at 3.2 Å from the substrate at subsite +1 (Figure 4). The residue is part of the conserved sequence region II of the α -amylase family (3, 43), but it is only conserved in CGTases. In α -amylases, for example, alanine, serine, threonine, and phenylalanine residues are found at the equivalent position (3). CGTase mutants of Ala230 have not been reported, but mutation of the equivalent alanine residue in *Bacillus licheniformis* α -amylase into a serine or glycine reduced the enzyme activity 50-fold (44). The saturation mutagenesis that we carried out on Ala230 in BC251 CGTase is in agreement with that result: most mutations at this position are harmful, as 70% of the clones obtained had less than 5% of wild-type cyclization activity. Only mutant A230V showed an increased hydrolytic activity (24-fold), while its disproportionation and cyclization activities were strongly decreased (100- and 20-fold, respectively; Table 3), making hydrolysis the main activity. From this we conclude that Ala230 is a residue that is absolutely critical for the transglycosylation activity of CGTase.

The strongly reduced transglycosylation activities of mutant A230V can be explained by the valine side chain, which likely hinders sugar binding at acceptor subsite +1 (Figure 4). All reactions catalyzed by CGTase start with substrate binding, glycosidic bond cleavage, and covalent glycosyl-enzyme intermediate formation (Figure 1). The efficiency of this step is governed by the productive binding of the -1 substrate residue and the scissile bond (5, 45). The next step is the attack of the C1 atom of the covalent reaction intermediate by a nucleophilic oxygen atom. In case of a transglycosylation reaction, this attack is done by the O4 atom of a sugar acceptor (5, 45). For this step, the correct orientation of the attacking +1 glucose residue in subsite +1 is critical. The larger valine side chain (A230V) may cause steric hindrance to the O3 atom of the +1 glucose and thus could interfere with the correct binding of the +1 glucose. Probably mutant A230V has a much higher K_M value for sugar acceptor substrates. However, due to the high hydrolytic activity toward the EPS substrate of the disproportionation reaction and the very low disproportionation activity of this mutant, we were not able to determine a K_M value for the acceptor substrate (maltose) of the disproportionation reaction. The reduced overall activities of mutant A230V may be explained by the effect on the initial substrate binding. However, the preference of mutant A230V for water over sugar acceptors [72 compared to 13 and 10 $\mu\text{mol}/(\text{min}\cdot\text{mg})$] can only be explained by an effect on the use of sugar acceptors in the second half of the reaction, showing that mutation A230V hinders the use of sugar acceptors. To explain the increased hydrolysis activity of mutant A230V, we may speculate that the valine side chain enhances the productive binding of a nucleophilic water molecule in the +1 acceptor binding site. A second explanation could be

that the covalent reaction intermediate is destabilized in the A230V mutant relative to wild-type CGTase and that the reaction intermediate collapses faster to form a hydrolysis product. However, the similarity of the A230V structure to that of wild-type CGTase, the similar (apolar) properties of alanine and valine, and the 4.4 Å distance to the covalent bond between Asp229 and the -1 glucose residue make this possibility not very likely.

Mutation A230V Provides Evidence for an Induced-Fit Mechanism. Mutant A230V has a much higher hydrolytic than transglycosylation activity [72 compared to 10–13 $\mu\text{mol}/(\text{min}\cdot\text{mg})$, respectively]. Because both reactions proceed via a covalent glycosyl-enzyme intermediate, these activities show that in the A230V mutant the covalent intermediate is still formed at a significant rate [at least 72 $\mu\text{mol}/(\text{min}\cdot\text{mg})$, which is equivalent to a k_{cat} of 90 s^{-1}]. However, the intermediate preferably reacts with water and not with an acceptor sugar.

For wild-type CGTase it has been argued that the transglycosylation activity of the enzyme is enhanced via an induced-fit mechanism upon binding of a carbohydrate residue in acceptor subsite +1 (16). Tyr195 and the glucoses bound at subsites -6, -3, and +1 play a key role in this induced-fit activation. Since a water molecule bound at subsite +1 is not able to produce this activation, this was suggested as an explanation for the low hydrolytic activity of CGTase (16).

The A230V mutation hinders acceptor sugar binding at subsite +1 (Figure 4), thereby interfering with such an induced-fit activation. This would result in a decreased transglycosylation activity, which is indeed clearly observed (Table 3). The strongly reduced transglycosylation activity of the A230V mutant, which is even much less than the hydrolytic activity of the mutant, provides thus biochemical evidence for the presence of an induced-fit mechanism in the second step of the transglycosylation reaction, in particular because the first step of the reaction (the formation of the covalent intermediate) takes place at a significant rate.

In contrast, the hydrolytic activity of the A230V mutant is strongly increased. The cause of this increased activity needs further study. For instance, it could be the result of enhanced productive binding of the nucleophilic water molecule in the +1 acceptor binding site, or the binding of a water molecule in the +1 subsite could activate the catalytic machinery for hydrolysis via a (different) induced-fit movement. Another explanation for the increased hydrolysis activity is the decreased cyclization and disproportionation activities, which gives the covalent glycosyl-enzyme intermediate a higher possibility to react with a water molecule, as the intermediate is degraded via either cyclization, disproportionation, or hydrolysis. In this case the increased hydrolytic activity is a consequence of a decrease in the competing cyclization and disproportionation activities.

CONCLUSIONS

Using error-prone PCR mutagenesis, we have identified several residues that are important for the transglycosylation reaction specificity of CGTase. After three rounds of mutagenesis the hydrolytic activity had increased 90-fold, yielding the highest hydrolytic activity ever reported for a CGTase. Furthermore, the much higher hydrolytic than transglycosylation activity of mutant A230V provided for

the first time biochemical support for a hypothesis that sugar acceptor binding at acceptor subsite +1 activates the catalytic machinery of glycosidic bond formation via an induced-fit mechanism. Such a mechanism can explain the high trans-glycosylation specificity of CGTase as it favors the use of glycosyl acceptors.

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